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FOOD TECHNOLOGY

Gastrorepair potential of functional fermented orange beverage against ethanol-induced gastric ulcer in rats

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ABSTRACT: Previous investigations have revealed that a functional fermented orange beverage presented in its composition different phenolic compounds, which through *in silico* investigation demonstrated to have biological effects of therapeutic importance as antioxidant, gastrorepair, and anti-ulcerative properties. Thus, this study confirmed *in vivo*, through a model of EtOH-induced gastric ulcers in rats, the beneficial properties indicated by the *in silico* tests. Gastric ulcer was induced by EtOH (intragastric) and was treated after 1 h with fermented orange beverage with and without *Mentha piperita* extract (0.5 mL/100 g w.b). Omeprazole was used as positive control. Histopathological evaluation revealed that EtOH administration resulted in the formation of gastric ulcers due to the reduction of the mucus layer, presence of hemorrhage, and infiltration of neutrophils in the stomach tissue of rats, and only treatment with omeprazole was able to reverse these changes. Additionally, EtOH administration altered the gastric juice volume and induced oxidative stress in the gastric tissue observed through the increase in lipid peroxidation (TBARS), reduction in the levels of non-protein thiols (NPSH), and alteration in the superoxide dismutase (SOD) activity. The ingestion of the fermented orange beverage increased NPSH levels and reduced changes in TBARS levels induced by ethanol. These findings suggested that the fermented orange beverage has antioxidant effects, as pointed out by *in silico* studies, but not gastrorepair and anti-ulcerative effects.
Key words: gastric ulcer, oxidative stress, antioxidants defenses, polyphenols, functional beverages.

Avaliação do potencial gastroreparador da bebida funcional fermentada de laranja contra úlcera gástrica induzida por etanol em ratos

RESUMO: Investigações anteriores revelaram que uma bebida funcional fermentada de laranja apresentou em sua composição diferentes compostos fenólicos, que através de investigações *in silico* demonstraram ter efeitos biológicos de importância terapêutica como propriedades antioxidantes, gastroreparadoras e antiulcerativas. Assim, o objetivo deste estudo foi confirmar *in vivo*, através de um modelo de úlcera gástrica induzida por EtOH em ratos, as propriedades benéficas indicadas pelos ensaios *in silico*. A úlcera gástrica foi induzida por EtOH (intragástrico) e tratada após 1 h com bebida fermentada de laranja com e sem o extrato de *Mentha piperita* (0,5 mL/100 g peso corporal). Omeprazol foi usado como controle positivo. A avaliação histopatológica revelou que a administração de EtOH resultou na formação de úlceras gástricas decorrentes da redução da camada de muco, presença de hemorragia e infiltração de neutrófilos no tecido estomacal de ratos, sendo apenas o tratamento com omeprazol capaz de reverter essas alterações. Além disso, a administração de EtOH alterou o volume do suco gástrico e induziu estresse oxidativo no tecido estomacal observado através do aumento da peroxidação lipídica (TBARS), redução dos níveis de tióis não-proteicos (SHNP) e alteração da atividade da superóxido dismutase (SOD). A ingestão da bebida fermentada de laranja aumentou os níveis de SHNP e reduziu as alterações nos níveis de TBARS induzidos pelo EtOH. Esses achados sugerem que a bebida fermentada de laranja apresenta efeitos antioxidantes, conforme apontado por estudos *in silico*, mas não efeitos gastroreparadores e antiulcerativos.
Palavras-chave: úlcera gástrica, estresse oxidativo, defesas antioxidantes, polifenóis, bebidas funcionais.

INTRODUCTION

Wine is one of the most consumed and preferred alcoholic beverages due to its organoleptic and health-promoting properties, and recently has been identified as a functional beverage (RADONJIĆ et al., 2020). In addition to the grape, this beverage can be produced from any sugary fruit as long as it is designated as fermented fruit (UMEH & UDEMEZUE, 2015). In the last decade, several studies have reported the production of fruit wine and the possible therapeutic properties

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(ESCUDERO-LÓPEZ et al., 2015; GONÇALVES et al., 2022; SHIRADHONKAR et al., 2014; UMEH & UDEMEZUE, 2015).

Brazil is considered the largest producer of oranges (FAO, 2021) and its different fruiting periods enable various harvests, thus avoiding the concentration of crops and reducing production costs throughout the year (PASSOS et al., 2018). Oranges are an abundant source of vitamin C and have considerable amounts of sugar, minerals, and bioactive compounds such as carotenoids, flavonoids, and essential oils (FERRARA et al, 2023). Thus, their use as raw materials to produce alcoholic beverages is an attractive option to add value to the product, diversify the market, and a solution to minimize the losses of these fruits in the fields or during transportation (FERRARA et al, 2023).

Excessive consumption of alcoholic beverages contributes to increased superoxide anions, hydroxyl radicals, and lipid peroxidation in the gastric mucosa, inducing intracellular oxidative stress that leads to the development of ulcerative gastric lesions (MOUSAVI et al., 2020). Gastric ulcer is a chronic disease characterized by an imbalance between protective agents, such as mucus, prostaglandins, bicarbonate and nitric oxide, and aggressive agents, such as pepsin and hydrochloric acid (HCl), in the gastric mucosa (BEIRANVAND, 2022; SHAKER et al., 2010).

Conversely, several studies have shown that moderate consumption, especially of wines, may have positive effects on health (KANG et al., 2023). A study carried out with healthy mice showed that the ingestion of an alcoholic fermented orange beverage (FOB) has greater protection against cardiovascular risk factors than orange juice (ESCUDERO-LÓPEZ et al., 2015). Another study reported that the regular consumption of low alcohol content orange beverages reversed metabolic parameters and modulated inflammatory response, lipid profile, and oxidative stress in rodents (ESCUDERO-LÓPEZ et al., 2016). These beneficial effects are related to the presence of phenolic compounds in these beverages and the mechanisms responsible for the potential health benefits of polyphenols are complex and generally attributed to their ability to directly eliminate free radicals and more recently to the up-regulation of endogenous antioxidant defenses (MITHUL ARAVIND et al., 2021).

The synthetic drugs used in the clinical treatment of gastric ulcers pose serious complications after prolonged use (FREEDBERG et al, 2017). Therefore, the discovery of natural compounds or nutritional strategies that can attenuate or prevent gastric ulcers are particularly relevant and of great interest to the food and pharmaceutical industry. In a recent study, we showed that functional FOB have phenolic compounds such as hesperidin, narirutin, and chlorogenic, caffeic, and ferulic acids in their composition (MASCARIN et al., 2023). These compounds through *in silico* studies have shown potential for biological effects of therapeutic importance, such as antioxidant, gastrorepair, and antiulcerative properties, being the *Mentha piperita* (peppermint) extract presented the greatest potential among the evaluated herbs for use in functional fermented beverages (MASCARIN et al., 2023).

Plant extracts have shown promising results in the treatment of ulcers by not only providing gastric protection but also gastric healing due to their antioxidant pharmacological properties (BEZERRA et al., 2009; FRANCISCO et al, 2014). The herb *M. piperita* L. has anti-inflammatory, antioxidant, and gastroprotective activity that is widely reported in the literature (GÓIS et al., 2016; GUL et al, 2015; URIBE et al., 2015). Also, their volatile aromatic profile allows them to be potentially included in beverages (SHIRADHONKAR et al., 2014), generating a differentiated product.

In this context, this study evaluated the gastrorepair, antiulcerative, and antioxidant effect of different functional FOB (with or without addition of *M. piperita* extract) in a model of EtOH-induced gastric ulcer in rats, aiming to confirm *in vivo* the beneficial properties previously indicated by the *in silico* tests.

MATERIALS AND METHODS

Fermented orange beverage (FOB) production with the addition of aromatic herbal extracts

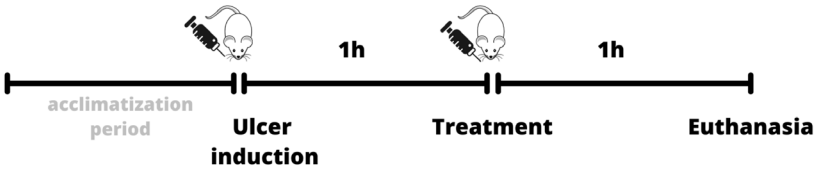
The FOB was produced according to MASCARIN et al. (2023). Oranges of the cultivar 'Valencia' (2018 harvest) were purchased in the city of Mata, southern Brazil, and the juice was extracted and filtered to remove the solid residue. The must was prepared for fermentation by adding 6% sodium metabisulfite (70 ppm) and left to rest for 60 min. Then, 230 $g.L^{-1}$ of sugar (26 ºBrix) was added, homogenized, and the pectinolytic enzymes (Lafazin Extract®, 3 $g\ hL^{-1}$; NutriStart®, 40 $g\ hL^{-1}$) were added. The yeast *Saccharomyces cerevisiae* (Blastosel Delta®, 40 $g\ hL^{-1}$) was inoculated and the alcoholic fermentation was carried out in 20 L polyethylene kegs at a controlled temperature (16 ± 2 ºC) and with decreased and stabilized total solid soluble to determine the endpoint of fermentation.

After fermentation, the temperature was maintained at 5 ºC for 48 h to separate the yeasts and other solids. The must was racked, filtered, and 50 ppm of 6% sodium metabisulfite was added and kept stabilized for three months with a controlled temperature of 16 ± 2 ºC.

The aromatic herb - *M. piperita* L. (leaves) - was purchased locally and in dry form, which is suitable for making teas. The hydro-alcoholic extract of aromatic herb (50% EtOH/H_2O) was prepared from equal volumes of 96 ºGL cereal alcohol and distilled water. Then, 100 g of dry matter from each plant was weighed and placed in maceration in 1 L of 50% hydroalcoholic solution, which was the volume necessary to fully cover the dry matter, resulting in an extract concentration of 10 g% (SHIRADHONKAR et al., 2014). The herb was kept in infusion in dark bottles at room temperature for 14 days to optimize the extraction of compounds. Afterward, the aromatic herbal extract was added to the fermented beverage (2.0%, v/v). The mixture remained stabilized for 2 months, in the dark, and at a controlled temperature of 16 ± 2 ºC (SHIRADHONKAR et al., 2014). The physical-chemical characterization of the beverages is available in MASCARIN et al. (2023).

Animals and induction of gastric lesions by EtOH

Adult male Wistar rats were provided by the Central Animal House of the Federal University of Santa Maria and all procedures adopted were approved by the institutional Animal Ethics Committee (protocol nº 5178300819). Animals were housed in standard polypropylene cages (four rats/cage) and kept under controlled temperature (22 ± 2˚C) and humidity (55 ± 5%) at a 12/12 h light/dark cycle with access to food and water ad libitum. Animals were divided into seven experimental groups (Figure 1). The control groups contained 4 animals per group (reduced number



Group	Ulcer induction	Treatment
Control	Received water (0.5 mL/100 g b.w.)	Received water (0.5 mL/100 g b.w.)
EtOH	Received ethanol (0.5 mL/100 g b.w.)	Received water (0.5 mL/100 g b.w.)
FOB	Received water (0.5 mL/100 g b.w.)	Received fermented orange beverage (FOB) (0.5 mL/100 g b.w.)
EtOH-FOB	Received ethanol (0.5 mL/100 g b.w.)	Received fermented orange beverage (FOB) (0.5 mL/100 g b.w.)
FOB-MP	Received water (0.5 mL/100 g b.w.)	Received FOB with *Mentha piperita* (MP) (0.5 mL/100 g b.w.)
EtOH-FOB-MP	Received ethanol (0.5 mL/100 g b.w.)	Received FOB with *Mentha piperita* (MP) (0.5 mL/100 g b.w.)
EtOH-OMEP	Received ethanol (0.5 mL/100 g b.w.)	Received omeprazole (30 mg/kg b.w.)

Figure 1 - Experimental design. EtOH: ethanol; FOB: fermented orange beverage; FOB-MP: fermented orange beverage with *Mentha piperita* extract; OMEP: omeprazole.

according to the 3Rs practice) and the other groups contained 6 animals each.

The dose of 0.5 mL/100g b.w of FOB was established based on the international recommendations which establishes the maximum volume administered by gavage in rats as being 1% of body weight, that is, 1 ml for every 100g b.w. (TURNER et al., 2011). Thus, in the present investigations, the animals received 0.5 mL/100g b.w of liquid (EtOH or water) in the gastric ulcer induction stage + 0.5 mL/100g b.w in the treatment stage. Omeprazol (OMEP) was used as a positive control (BOLIGON et al., 2014) as it is a standard drug used for the clinical treatment of gastric ulcers.

After 7 days of acclimatization, rats were fasted for 12 h to ensure an empty stomach and efficient induction of gastric lesions. Gastric lesions were induced by an oral administration of 75% EtOH (0.5 mL/ 100 g b.w.) (BOLIGON et al., 2014). One hour after EtOH administration, the animals received the treatments (water, FOB, FOB-MP, or OMEP) by gavage. After another 1 h, animals were anesthetized with isoflurane and euthanized by cardiac puncture (Figure 1). Blood samples were collected for determination of serum glucose and the stomach of each rat was quickly removed for determination of gastric juice/secretion volume. The weight of the stomach (without gastric juice) and of the liver were determined. After, each stomach was dichotomized, one part was immersed in 10% formalin solution for histopathological analyses, whereas the other part was homogenized in 0.01 M phosphate buffered saline with 0.136 M NaCl (pH 7.4). One part of the homogenate was used to assess the levels of non-protein thiol groups (NPSH) and thiobarbituric acid-reactive substances (TBARS). Another part of the homogenate was centrifuged at 3500 rpm for 10 min to yield supernatant that was used to determine antioxidant enzyme activities and protein levels.

Histological analysis

Samples of stomach tissue fixed in 10% buffered formalin solution were processed and paraffin embedded. The histological sections were processed by a Thermo Scientific™ HM 325 rotary microtome with a thickness of 5 µm, in order to enable the production of two slides per section, which were later stained with Goldner-Masson trichrome. Each microscopic slide was evaluated in 10 histological fields using an Axio Scope A1 microscope coupled to an Axiocam 105 color camera (ZEISS®, Germany) and directly linked to software, which allows recording high-quality photos and recording them in sequence. Through the ImageJ program, random measurements were established throughout the entire sample in order to measure the thickness of the mucous, integrity of the gastric mucosa layer, hemorrhage, as well as neutrophil infiltration.

Markers of oxidative stress

In the homogenate of the stomach tissue, lipid peroxidation was estimated by the measurement of TBARS using a standard curve of 1,1,3,3-tetraethoxypropane (OHKAWA et al, 1979). To the determination of NPSH levels, the homogenate fraction was deproteinized with 10% trichloroacetic acid (1:1 v/v), and NPSH content were determined as described by Ellman (ELLMAN, 1959) using a standard curve of cysteine.

Antioxidant defense system

Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the auto-oxidation of epinephrine to adrenochrome at an alkaline pH (MISRA & FRIDOVICH, 1972), and catalase (CAT) activity was determined using hydrogen peroxide (H_2O_2) as substrate (AEBI, 1984). The pseudo-first order reaction constant (k) of the decrease in H_2O_2 absorption at 25˚C was determined, and the activity was expressed as k/ug protein.

Protein determination

Protein was measured in homogenate and supernatant for the normalization of oxidative status analyses using bovine serum albumin as the standard (LOWRY et al., 1951).

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Duncan's post hoc test when appropriate. Data that did not meet the ANOVA assumptions were analyzed by Kruskal Wallis analysis, followed by multiple comparison tests. Results were expressed as the mean ± SEM and differences were considered statistically significant when $P \leq 0.05$. Data were analyzed using the Statistica® V.7 software system (Statsoft Inc., 2004).

RESULTS AND DISCUSSION

In the present study, we investigated the gastrorepair effects of a functional FOB with or without addition of *M. piperita* extract in a model of gastric ulcers induced by EtOH in rats, aiming to confirm *in vivo* our previous findings that indicated beneficial properties through *in silico* tests (MASCARIN et al., 2023).

Body weight, organ weight, and serum and stomach parameters

The experimental groups were homogeneous when considering the basal body weight of the animals (Figure 2A; $P \geq 0.05$). Acute administration of ethanol or the different treatments did not change the weight of the liver or the stomach, nor did it alter the levels of serum glucose (Figure 2B – D, respectively; $P \geq 0.05$). These results were expected since the time between the beginning of the experimental protocol and the euthanasia of the animals is extremely short (2 hours in total). Similarly, Da Luz and collaborators also did not observe changes in body weight and organ weight of animals that received ethanol and/or plant extracts rich in phenolic compounds (DA LUZ et al., 2021).

Conversely, the induction of gastric ulcer through the administration of ethanol induced an increase in gastric juice volume (Figure 2E; $P \leq 0.05$). This increase in the volume of gastric juice was already expected, since it is already well known that in the stomach, alcohol can promote damage to the mucosa, influencing greater secretion of acid (BEIRANVAND, 2022; HUNT et al., 2015). The FOB and FOB-MP treatment in rats with gastric ulcer does not change the volume of gastric juice when compared to the EtOH group (Figure 2E; $P \leq 0.05$). It has already been reported that the beneficial activity of certain medicinal plants in the treatment of gastric ulcer was not related to anti-secretory mechanisms (LI et al., 2008; NAGULSAMY et al, 2015; SCHUBERT & PEURA, 2008). Although the current therapy for the treatment of gastric ulcers consists of the use of drugs with anti-secretory

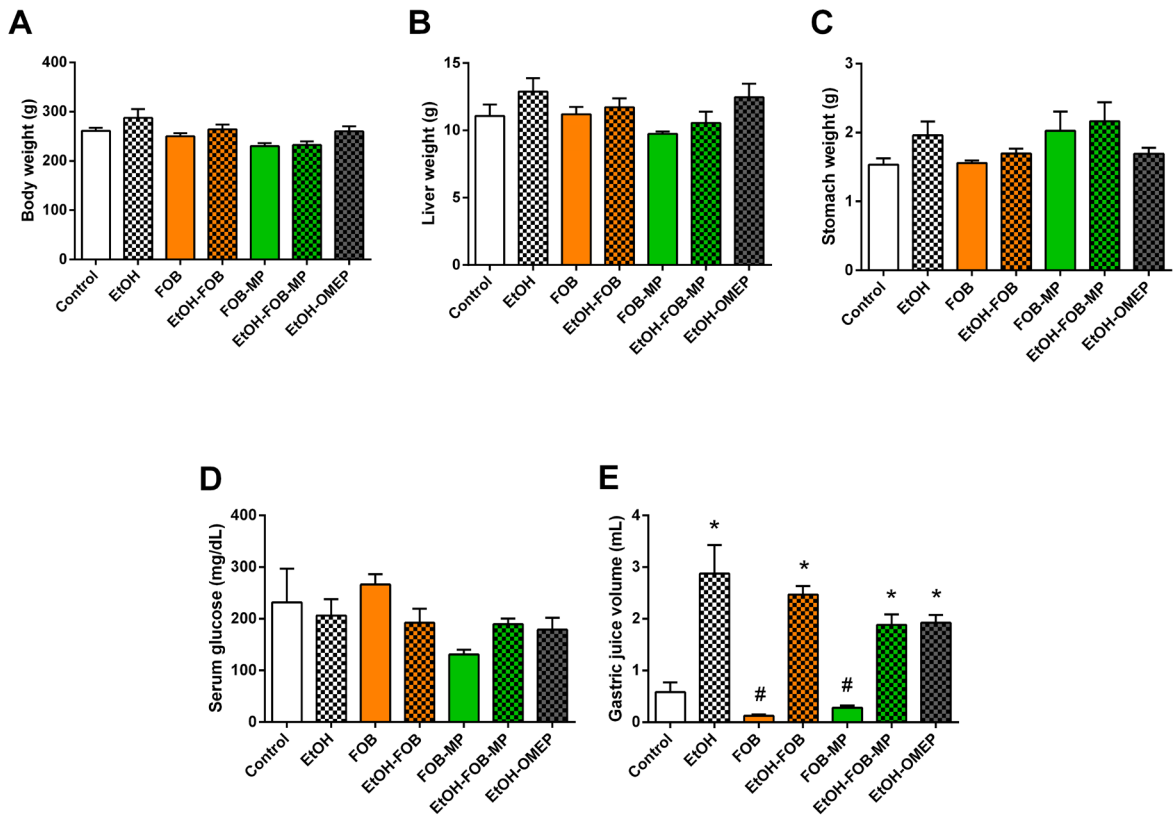


Figure 2 - Body (A), liver (B), and stomach (C) weights, serum glucose (D) and gastric juice volume (E) of rats with gastric ulcer induced by ethanol and treated with water, FOB, FOB-MP, and omeprazole. Data are shown as the means ± S.E.M. Tested by ANOVA followed by post hoc Duncan's test. *Different from the control group ($P \leq 0.05$). #Different from EtOH group ($P \leq 0.05$). Control group: without gastric ulcer; EtOH group: with gastric ulcer; FOB group: without gastric ulcer and treated with fermented orange beverage; EtOH-FOB: with gastric ulcer and treated with fermented orange beverage; FOB-MP: without gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-FOB-MP: with gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-OMEP: with gastric ulcer and treated with omeprazole.

activity, this strategy can cause hypergastrinemia in chronic users, because once treatment is interrupted, hypergastrinemia is responsible for a rebound effect on gastric secretion (BEIRANVAND, 2022; HUNT et al., 2015). Thus, new drugs or therapeutic strategies with no anti-secretory action are desirable, as they prevent microbial growth and hypergastrinemia in patients who use it chronically.

Effect of fermented orange beverage on EtOH-induced gastric damage

EtOH can be used to induce experimental gastritis and gastric ulceration (BEIRANVAND, 2022; HUNT et al., 2015). EtOH is known to penetrate the gastric mucosa, causing membrane damage, erosion of gastric cells, impairment in proton (H^+) pumping into the gastric lumen, features most likely to precede cell death in gastric mucosal cells (BEIRANVAND, 2022). In this study, the intragastric administration of EtOH induced the formation of gastric ulcer evidenced by changes in the thickness of the mucus layer, highlighting ulcerative lesions and reducing the protective layer of mucus (Figure 3). Farther, histopathological evaluations revealed erosion of gastric mucosal epithelium, hemorrhage, and inflammatory infiltration of neutrophils (EtOH group, Table 1), which is in accordance with previous reports of this model (BOLIGON et al., 2014; DA SILVA

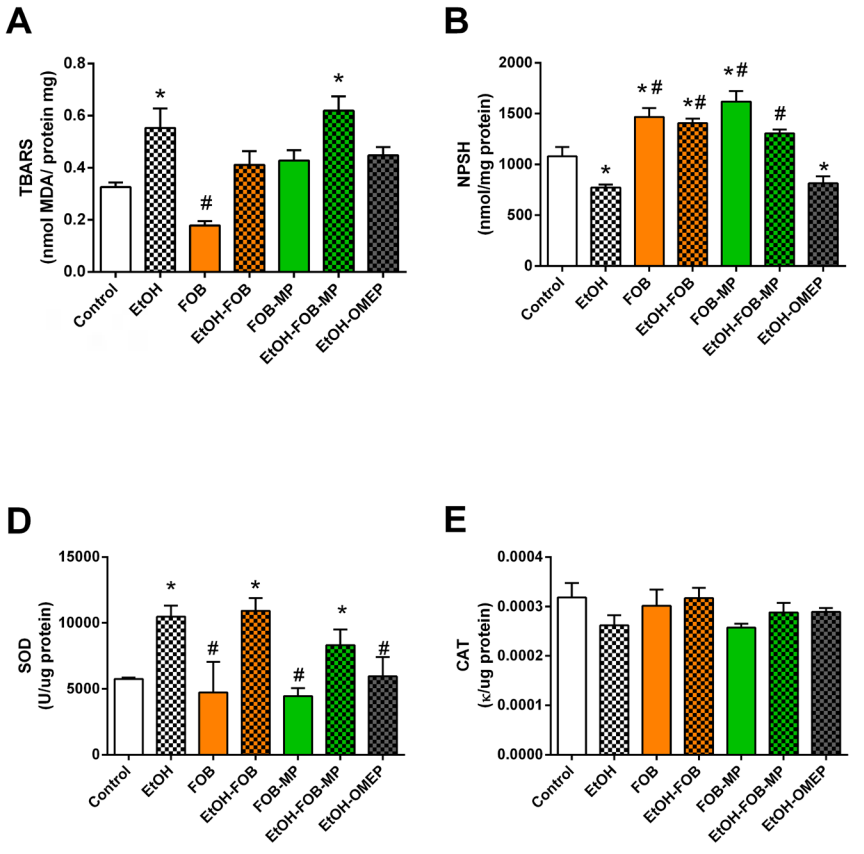


Figure 3 - Evaluation of the oxidative status of the stomach tissue through the levels of lipid peroxidation (A), non-protein thiols (B), and superoxide dismutase (C) and catalase (D) activities. Data are shown as the means ± S.E.M. Tested by ANOVA followed by post hoc Duncan's test. *Different from the control group ($P \leq 0.05$). #Different from EtOH group ($P \leq 0.05$). Control group: without gastric ulcer; EtOH group: with gastric ulcer; FOB group: without gastric ulcer and treated with fermented orange beverage; EtOH-FOB: with gastric ulcer and treated with fermented orange beverage; FOB-MP: without gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-FOB-MP: with gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-OMEP: with gastric ulcer and treated with omeprazole.

Table 1 - Histopathological indexes of stomach from rats treated with ethanol and/or fermented orange beverages or omeprazole.

	Hemorrhage	Erosion of gastric mucosal epithelium	Neutrophil infiltrate
Control	-	†	-
EtOH	††	††	††
FOB	††	††	†
EtOH-FOB	††	††	††
FOB-MP	††	††	†
EtOH-FOB-MP	††	††	†
EtOH-OMEP	†	†	†

Gastric tissue with no negative features was given a score of 0 (-). Gastric tissue with mild histopathological damage was given a score of †. Those with moderate and severe negative features were given a score of †† and †††, respectively. Results were expressed as a histopathological score. Control group: without gastric ulcer; EtOH group: with gastric ulcer; FOB group: without gastric ulcer and treated with fermented orange beverage; EtOH-FOB: with gastric ulcer and treated with fermented orange beverage; FOB-MP: without gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-FOB-MP: with gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-OMEP: with gastric ulcer and treated with omeprazole.

et al., 2020). EtOH increases vascular permeability and exposes gastric mucosa to the proteolytic and hydrolytic actions of pepsin and HCl, besides it causes blood flow stasis and leading to vascular damage and necrosis (ADINORTEY et al., 2013).

Treatment with FOB or FOB-MP did not reduce the ulcer induced by EtOH, as the ulcerative changes on the mucosal surface, hemorrhagic and neutrophil infiltrate are similar to the group that received only EtOH (gastric ulcer group, Table 1). The lack of effect may be related to the alcohol content of the fermented beverages used as a treatment in the present study – 16.2% (v/v) (MASCARIN et al., 2023). Since, surprisingly, the animals without gastric ulcer induction by EtOH and that received only FOB or FOB-MP also showed a reduction in the protective layer of mucus (Figure 3) and presented hemorrhage and erosion of the gastric epithelium, but do not infiltration of inflammatory cells (neutrophils) (Table 1). These results demonstrated that even much lower alcohol concentrations than those used for the induction of the model (75%) can have a negative impact on the gastric mucosa. Conversely, the lack of results may also be related to the type of experimental protocol used in this study (gastrorepair acute protocol). Possibly a gastroprotection protocol with orange fermented beverage treatment for a longer period, eg. 2 weeks, before ulcer induction could result in a different outcome. This gastroprotection protocol was performed by DA SILVA et al., (2020), who administered blueberry extract to the animals for 14 days before gastric ulcer induction and observed a decrease in the ulcer index and preservation of the integrity of the gastric mucosa.

The gastric mucosa of animals that received OMEP showed little preservation of the histological aspects and were slightly more preserved than the EtOH group (Figure 3 and Table 1). OMEP is a proton pump inhibitor that has been widely used in the treatment and prevention of gastrointestinal disorders as gastric ulcers (BEIRANVAND, 2022) and was used as a positive control in this study. These results demonstrated that the induction of gastric ulcer was very aggressive in this study, since even a drug widely used in the clinic for this purpose was not able to completely treat the effects resulting from the administration of EtOH.

Effect of fermented orange beverage on EtOH-induced changes in gastric oxidative status and antioxidant defense

The histopathological damage is related with overproduction of reactive oxygen species (ROS), lipid peroxidation, depletion of NPSH as glutathione (GSH), as well as, impairment in the antioxidant defense system that also contribute to the EtOH-induced gastric damage and which plays a crucial role in the pathogenesis of gastric ulcer (MOUSAVI et al., 2020; DA SILVA et al., 2020). In this study, the EtOH administration caused an increase in ROS production leading to lipid peroxidation – evaluated through TBARS levels (Figure 4A; $P \leq 0.05$). In response to elevated ROS levels, the stomach tissue of rats with untreated gastric ulcer showed a decrease in its non-

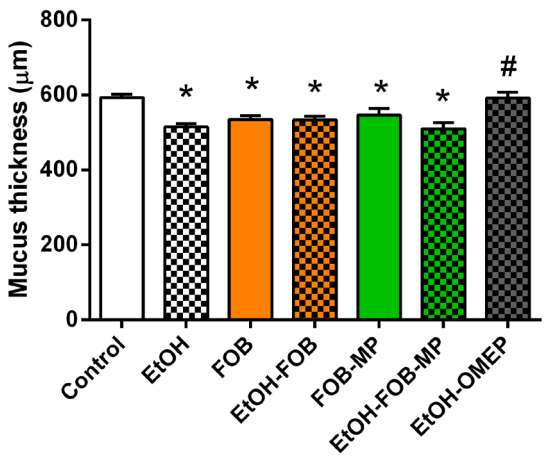


Figure 4 - Evaluation of the mucus layer in the stomach of rats with gastric ulcer induced by ethanol and treated with water, FOB, FOB-MP, or omeprazole. Data are shown as the means ± S.E.M. Tested by ANOVA followed by post hoc Duncan's test. *Different from the control group ($P \leq 0.05$). #Different from EtOH group ($P \leq 0.05$). Control group: without gastric ulcer; EtOH group: with gastric ulcer; FOB group: without gastric ulcer and treated with fermented orange beverage; EtOH-FOB: with gastric ulcer and treated with fermented orange beverage; FOB-MP: without gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-FOB-MP: with gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-OMEP: with gastric ulcer and treated with omeprazole.

enzymatic antioxidant defenses, evaluated through NPSH content and compared to the control group (Figure 4B; $P \leq 0.05$), indicating a reduction in GSH levels, the major non-protein thiol in mammalian cells and tissues. GSH is a tripeptide that acts on the primary antioxidant defense system and its decrease has been used as a marker of oxidative stress (ZITKA et al., 2012).

Additionally, EtOH administration significantly increased SOD activity in the stomach of the animals in the experimental conditions used in this protocol (Figure 4C, respectively; $P \leq 0.05$). This increase indirectly demonstrates an increase in the production of superoxide anion after EtOH administration. The release of superoxide anion in this type of injury may be related to the production of acetaldehyde, formed by the action of alcohol dehydrogenase on EtOH (BEIRANVAND, 2022). Acetaldehyde serves as a substrate for xanthine oxidase (a key enzyme in the metabolism of purines), which produces free radicals - among them superoxide (HALLIWELL & GUTTERIDGE, 1995).

Previous reports confirm that EtOH increases superoxide anion and hydroxyl radical production by neutrophils and these ROS cause lipid peroxidation in the gastric mucosa (DA LUZ et al., 2021; MOUSAVI et al., 2020; DA SILVA et al., 2020). These results showed that EtOH administration caused overproduction of ROS that overloaded enzymatic and non-enzymatic antioxidant defense systems, leading to oxidative stress that contributed to gastric mucosal damage, as observed in this study.

The CAT activity, which is important for the decomposition of H_2O_2, was not altered by the administration of EtOH (or any treatment) (Figure 4D). We believed that the levels of H_2O_2 generated after the decomposition of the superoxide anion by SOD were low, so the most appropriate would be to evaluate the activity of glutathione peroxidase (GPx), which acts at low levels of H_2O_2 (HALLIWELL & GUTTERIDGE, 1995), but unfortunately it was not possible to evaluate this enzyme in the present study (limitation).

Considering that oxidative stress is a key factor in the development of gastric lesions resulting from EtOH administration, the use of antioxidants has been proposed as therapeutic agents to protect gastric damage (BEIRANVAND, 2022; DA SILVA et al., 2020; MOUSAVI et al., 2020). Here, the treatment with FOB was able to reduce oxidative stress in stomach tissue by partially reducing lipid peroxidation and increasing non-enzymatic antioxidant defenses (Figure 4A and 4B, respectively; $P \leq 0.05$). This protective effect of FOB may be related to the presence of phenolic compounds in this beverage, derived from the fermentation of orange juice. FOB had in its composition phenolic acids (caffeic, ferulic, and chlorogenic acid) and flavonoids (rutin, narirutin, and hesperidin), being the latter group the one with the highest concentration (MASCARIN et al., 2023).

The beneficial action observed in the present study can be related to the direct free radical scavenging capacity of flavonoid polyphenols, which has been mainly attributed to the presence of catechol groups in ring B, double bonds and hydroxyl substitutions in the aromatic ring (WANG et al., 2017; OGAWA et al., 2011).

The FOB direct antioxidant capacity had already been observed in our *in vitro* studies (MASCARIN et al., 2023). In these studies, we also observed that FOB showed greater antioxidant direct capacity (assessed by the DPPH method) than FOB-MP. Additionally, FOB showed higher levels of ferulic acid and hesperidin than FOB-MP, in addition to the

presence of rutin that was not found in the beverage added with *M. piperita* extract (MASCARIN et al., 2023). These differences in the antioxidant capacity and phytochemical composition between FOB and FOB-MP may be the reason why we observed a beneficial effect against oxidative stress in animals that were treated with FOB, but not in those that received FOB-MP.

In addition to the direct free radical scavenging capacity, the beneficial effect of FOB treatment observed in animals with gastric ulcer is also due to the improvement of endogenous non-enzymatic antioxidant defenses. We believe that the compounds present in FOB may also have modulated enzymes related to the synthesis and/or recycling of GSH, strengthening non-enzymatic antioxidant defenses. This hypothesis can be confirmed by observing the increase in NPSH levels in all animals that received FOB (Figure 4B; $P \leq 0.05$), being a direct reflection of the increase in GSH levels. FOB-MP also seems to perform a similar action only in animals without gastric ulcer (Figure 4B; $P \leq 0.05$).

Recently, DA SILVA and collaborators demonstrated that plant extracts rich in phenolic compounds were able to increase the GSH/GSSG ratio by modulating the activity of enzymes in the GSH synthesis/recycling cycle, such as GPx, glutathione reductase (GR), and glutathione-s-transferase (GST) (DA SILVA et al., 2020). Unfortunately, we did not determine the activity of these enzymes and this is a limitation of our study. Conversely, natural compounds, such as phenolic compounds, can also present some degree of toxicity depending on the exposure conditions and dose (OTTOBONI, 1991) and induce defense or detoxification mechanisms in cells causing a compensatory increase of detoxification molecules such as GSH. In the present study, it is believed that the increase in NPSH levels is not related to the toxicity mechanism, since the orange fermented beverage dose administered to the animals was extremely low (FOB was in the range of 2.39 µg/g b.w., and FOB-MP was in the range of 2.58 µg/g b.w). Studies with different phenolic compounds (hesperetin, naringenin, baicalein, galangin, genistein, quercetin, etc.) have shown that these compounds have low cytotoxicity (> 500 µg/mL for 50% cell death) (SO et al., 1996; HUANG et al., 1999).

The reduction in lipid peroxidation in the stomach tissue observed in the animals that received FOB may be, in addition to the improvement in GSH levels, due to a direct antioxidant effect of the phenolic compounds, which helped the antioxidant defenses in neutralizing the excess of ROS and; consequently, in reducing lipid peroxidation. Despite this, FOB treatment failed to reduce ethanol-induced tissue damage, and this lack of gastrorepair effect may be related to the alcohol content of this beverage and/or the experimental protocol (gastrorepair protocol) used in the present investigation.

The FOB and FOB-MP treatments did not prevent changes in SOD activity when compared to the EtOH group (Figure 4C; $P \leq 0.05$). This non-modification of SOD activity may be due to EtOH administration in these groups. Despite having improved antioxidant status, by increasing NPSH levels, this was not enough to eliminate all the superoxide produced as a result of the decomposition of EtOH by alcohol dehydrogenase (BEIRANVAND, 2022).

The treatments with OMEP causes reduction in oxidative stress, evidenced by lower levels of TBARS and, consequently, SOD activity when compared to the EtOH group (Figure 4A and 4C, respectively; $P \leq 0.05$). This is possibly due to a lesser degree of damage to stomach tissue as pointed out by histological findings (Figure 2 and Table 1), with lower production of ROS, which consequently requires less adaptive changes of the antioxidant defense system, and not to an antioxidant effect of OMEP, since this drug did not improve antioxidant status *per se* (did not increase NPSH levels when compared to the control; Figure 4D).

In this investigation, we referred to the fermented orange as functional since so-called "functional beverages" provide important bioactive compounds for maintaining health and/or contributing to the prevention and treatment of chronic diseases (BULMAN et al., 2021; CONG et al., 2020; GONÇALVES et al., 2022). The "functional properties'' can be characterized by high polyphenol content, antioxidant potential, probiotics, and other characteristics (BULMAN et al., 2021; CUVAS-LIMÓN et al., 2022; PINTO & VILELA, 2021), even alcoholic beverages such as wine and beer have already been identified as functional beverages (RADONJIĆ et al., 2020). FOB had already demonstrated good antioxidant capacity in different *in vitro* evaluations (MASCARIN et al., 2023) and now it has demonstrated this property *in vivo*, which confirmed that FOB is indeed a functional beverage.

CONCLUSION

In this investigation, treatment with FOB showed an antioxidant action *in vivo* in an EtOH-induced gastric ulcer model, confirming previous

findings in *silico* studies. The phenolic composition, mainly flavonoids, is possibly the determining factor for FOB to have an antioxidant effect and FOB-MP not. The experimental protocol used in this investigation and/or the presence of high EtOH concentration in FOB and FOB-MP may have been the factors for the absence of gastrorepair and antiulcerative effects. Gastroprotection trials may have more promising results than the gastrorepair trials we performed. The consumption of this functional beverage, preferably dealcoholized, can be further investigated as a nutritional adjuvant strategy in the prevention of diseases with the involvement of oxidative stress.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

L.G.M.: doctoral student, participated in the entire analyses, writing and revision of the study. F.W.F., F.Z.R., J.R.P.G, and W.N.P.: participated in the execution of the *in vivo* experiment. J.O.F.S. and S.T.C: performed histopathological analysis and data interpretation. J.R.B. and C.C.D.: performed the biochemical analyses. L.F.B., S.S., and C.K.S.: participated in the entire analyses, writing and revision of the study.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The authors declare that this study was submitted for evaluation by the Ethics Committee of the Universidade Federal de Santa Maria (UFSM) and was approved under number 5178300819 (ID 002808).

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